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Original Article

Patterns of dystrophin expression in developing, adult and regenerating tail skeletal muscle of Amphibian urodeles

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ABSTRACT The patterns of expression of dystrophin were investigated by indirect immunofluorescence and by immunoblotting in developing, adult and regenerating tail skeletal muscle of newts *Pleurodeles waltl* and *Notophthalmus viridescens*. In this study, a monoclonal antibody H-5A3 directed against the C-terminal region (residues 3357-3660) and a polyclonal antibody raised to the central domain (residues 1173-1738) of the chicken skeletal muscle dystrophin were used. Western blot analysis showed that these antibodies recognized a 400 kDa band of dystrophin (and may be of dystrophin-related protein) in the adult muscle tissues and in newt tail regenerates. During skeletal muscle differentiation or epimorphic regeneration (blastema), anti-dystrophin immunoreactivity gradually accumulated over the periphery of the myofibers. Dystrophin and laminin were first and concomitantly observed at the ends of the newly formed myotubes where they were anchored on connective tissue septa or bone processes by dystrophin-rich myotendinous structures. It is noteworthy that neuromuscular junctions, which most probably also contain dystrophin, are established in urodeles near the ends of the myofibers as shown by histochemical localization of AChE activity or fluorescent bungarotoxin detection of AChRs. In the stump transition zone close to the tail amputation level where tissue regeneration of injured muscle fibers took place, dystrophin staining located on the cytoplasmic surface of myofibers progressively disappeared during the dedifferentiation process which seemed to occur during muscle regeneration as suggested by electron microscopy. Furthermore, double labeling experiments using anti-dystrophin and anti-laminin antibodies showed a good correlation between the remodeling processes of the muscle fiber basal lamina and the loss of dystrophin along the sarcolemma of damaged and presumably dedifferentiating muscle cells.

KEY WORDS: *dystrophin, skeletal muscle, epimorphic and tissue regeneration, differentiation, dedifferentiation, Amphibian urodeles*

Introduction

Adult amphibian urodeles — such as newt, salamander or axolotl — are the only vertebrates that have the remarkable capacity to regenerate the missing part of their appendages (limb or tail) after amputation, by forming a blastema under the wound epithelium (for review, see e.g. Wallace, 1981). This regeneration blastema, which is roughly the equivalent of an embryonic bud, is composed of histologically undifferentiated mesenchymal cells which originate at least in part from dedifferentiation of stump mesodermal tissues, including skeletal muscle (Hay, 1962), near the site of amputation, in a way that remains not fully understood (Ferretti and Brockes, 1991). Regarding the skeletal muscle, two modes of regeneration are in fact temporally and spatially distinct in this system (Carlson, 1979; Thouveny *et al.*, 1991): (1) a tissue mode that repairs the damaged muscle fibers in the stump region (or transition zone) adjacent to the amputation plane; and (2) an epimorphic mode that occurs within the regenerate itself.

The epimorphic muscle regeneration, which proceeds in a rostrocaudal differentiation gradient (Iten and Bryant, 1976), closely resembles the normal myogenic pattern as shown by morphological studies (Hay, 1962; Lentz, 1969; Carlson, 1979; Khrestchatisky *et al.*, 1988). This muscle regeneration mode, in which extracellular matrix components — especially tenascin — may be involved

Abbreviations used in this paper: Nv, *Notophthalmus viridescens*; Pw, *Pleurodeles waltl*; NMJs, neuromuscular junctions; MTJs, myotendinous junctions; AChE, acetylcholinesterase; AChRs, acetylcholine receptors.

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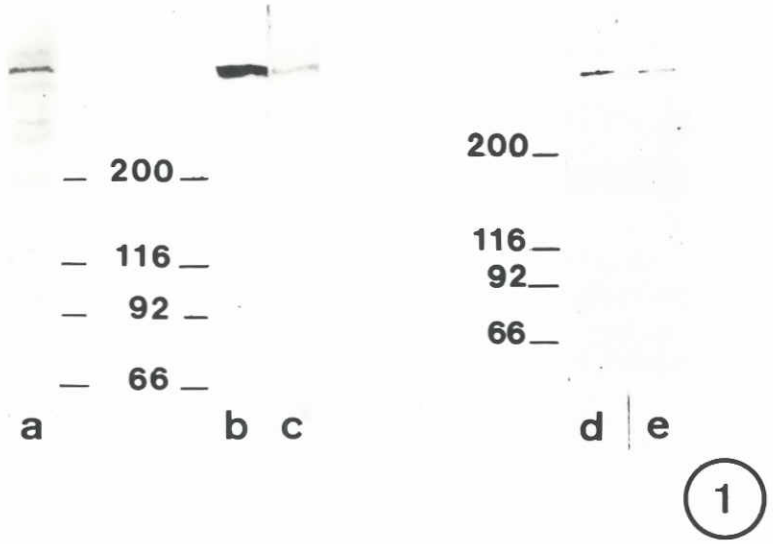


Fig. 1. Immunoblots with anti-dystrophin antibodies. *Samples of muscle tissues* **(lanes a,b,c)** *and tail regenerates* **(lanes d,e)** *from adult Pleurodeles waltl were resolved on 7% (lanes a,b,c) or 5-13% (lanes d,e) SDS/PAGE, and then transferred to nitrocellulose. The blots were probed with anti-dystrophin PAb (lane a) or H-5A3 MAb (lanes b,c,d,e). Lanes a,b: skeletal muscle; lane c: smooth muscle from gut; lanes d,e: tail regenerates of 5 or 4 weeks. Mr x10^{-3} standards are indicated: myosin (200 kDa); b galactosidase (116 kDa); phosphorylase b (92 kDa) and BSA (66kDa).*

(Arsanto *et al.*, 1990), leads to the reestablishment of the metameric organization (Iten and Bryant 1976) and innervation (Thouveny *et al.*, 1991; Arsanto *et al.*, 1992) pattern of normal adult newt tail skeletal musculature.

On the other hand, recent immunolocalization and cell fractionation studies (reviewed e.g. in Hoffman and Kunkel, 1989; Léger *et al.*, 1991) have shown that dystrophin — the defective gene product in Duchenne and Becker Muscular Dystrophies (DMD/BMD) (Hoffman *et al.*, 1987) — is a cytoskeletal protein tightly associated with the cytoplasmic face of normal mature muscle fibers. This molecule the expression of which in muscle cells is developmentally regulated (Lev *et al.*, 1987; Nudel *et al.*, 1988; Hagiwara *et al.*, 1989; Chelly *et al.*, 1990) is thought to play a crucial role, not only in the stabilization of myofiber membrane, but also in the organization of myotendinous and neuromuscular junctions (for review, see e.g. Léger *et al.*, 1991).

We therefore were interested in analyzing the expression pattern of dystrophin(s) in developing, mature and tissue or epimorphic regenerating skeletal muscle of caudal appendage of urodeles.

In this work, double immunostaining experiments with anti-dystrophin and anti-laminin antibodies were used to study, in the transition zone, the remodeling processes of muscle fiber basal lamina in correlation with the loss of dystrophin during the dedifferentiation stages of muscle tissue regeneration.

On the other hand, the localization of neuromuscular synaptic sites — which showed dystrophin immunoreactivity — was checked by histochemical or fluorescence detection of acetylcholinesterase activity (AChE) or acetylcholine receptors (AChRs), respectively.

Part of the results reported here were presented at the last Congress on Neuromuscular Diseases organized by the «Association Française contre les Myopathies» (AFM) (Arsanto *et al.*, 1991).

Results

Western blot analysis

Polyclonal antiserum raised against the central domain of the dystrophin recognized a band of 400kDa in samples of muscle tissues, especially in skeletal muscle from adult Pw (Fig. 1, lane a).

On Western blots with H-5A3 MAb directed against the C-terminal domain of chicken dystrophin, dystrophin immunoreactivity also appeared as a protein of 400kDa in adult skeletal and smooth (gut) muscle tissue extracts (Fig. 1, lanes b and c, respectively).

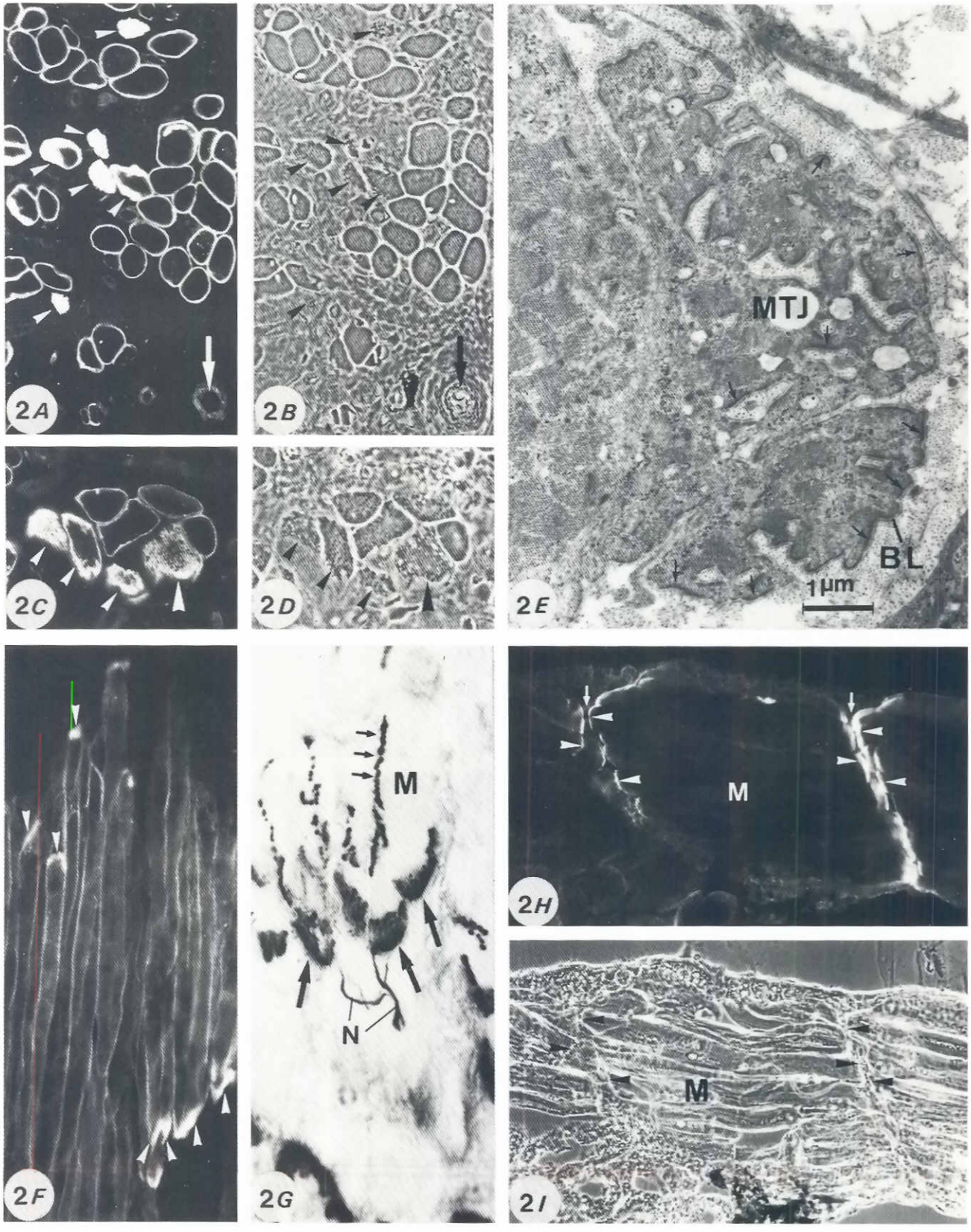
Furthermore, in samples from 3 to 4 week-old newt tail regenerates, a 400kDa band was also clearly stained by these anti-dystrophin antibodies, e.g. H-5A3 MAb (Fig. 1, lanes d,e), although weaker than in adult skeletal muscle (Fig. 1, lane b).

Immunofluorescence, histochemical and electron microscopy

Skeletal muscle in adult newt and larva

With anti-dystrophin H-5A3 MAb (Fig. 2A-B,C-D,F) or PAb (results not shown), cryostat cross (Fig. 2A-B,C-D) and longitudinal (Fig. 2F) sections showed labeling of dystrophin at the periphery of skeletal

Fig. 2. Localization of MTJs and NMJs in skeletal muscle of urodeles. *(A-C) Anti-dystrophin labeling with H-5A3 MAb in cross-sections through adult skeletal muscle fibers of Nv.* **(A)** *The most intensely reactive areas visualize cross-sections through the myotendinous junctions (MTJs, arrowheads). Note also the staining of tunica media of a blood vessel (arrow).* **(B)** *Phase-contrast image of (A). X200.* **(C)** *Another view of myofibers in cross-section showing the detailed structure of dystrophin-positive MTJs (arrowheads).* **(D)** *Phase-contrast image of (C). X300.* **(E)** *Electron micrograph of cross-section through subterminal regions of adult muscle fibers. The plane of section nearly coincides with the insertion zone of fibers where MTJs can be seen with extensive cell membrane foldings. Note the highly contrasted subplasmalemmal sites (arrows) at which thin myofilaments are linked to the cell membrane. BL: basal lamina. X1200.* **(F)** *Anti-dystrophin labeling in longitudinal section through adult skeletal muscle fibers of Nv. Immunoreactivity is observed at the periphery of myofibers and more intensely at their terminal regions. X200.* **(G)** *Histochemical detection of AChE at the neuromuscular junctions (NMJs) of longitudinally sectioned myofibers of adult Nv. AChE-reactive regions visualizing the NMJs are mainly observed near the ends (large arrows) of myofibers (M). AChE-positive synaptic dots are also seen (small arrows) on the lateral surface of fibers. N: Silver-stained nerve fibers. X250.* **(H-I)** *Part of longitudinal section through myotoma of Pw larva.* **(H)** *Intense dystrophin labeling with H-5A3 MAb is observed at the terminal regions (arrowheads) of myofibers (M) involved in anchorage of fibers on myoseptal connective tissue (arrows).* **(I)** *Phase-contrast image of (H). X150.*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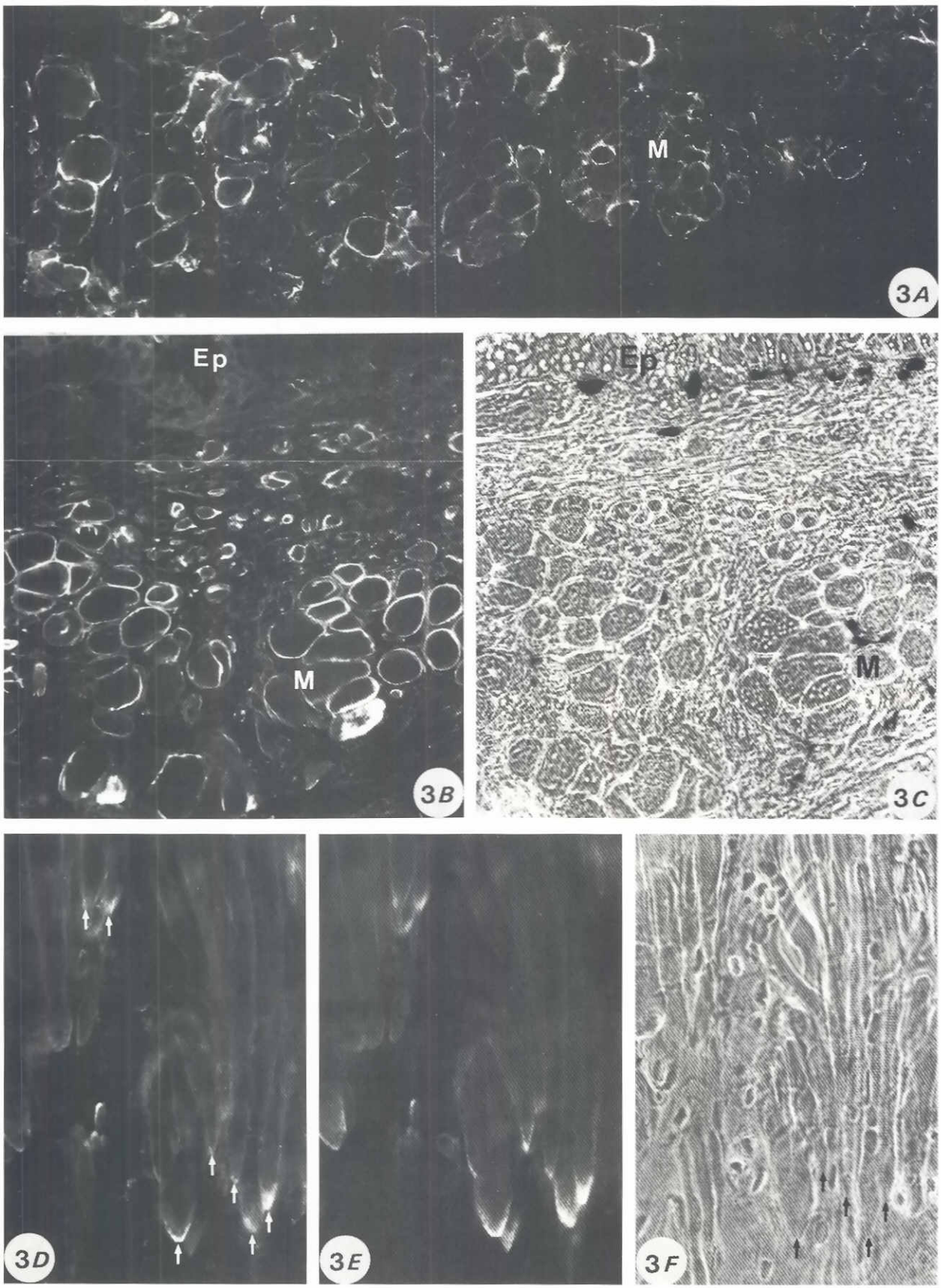
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muscle fibers. However, the terminal regions of the myofibers, i.e. the myotendinous junctions (MTJs) implicated in the anchorage of thin myofilaments on myoseptal connective tissue or on bone processes occurred as the most intensely reactive structures of myofibers in larva (Fig. 2H) as well as in adult newt (Fig. 2F). Highly stained areas seen in muscle cross sections (Fig. 2A,C, arrowheads) correspond to these dystrophin-rich myofiber regions in which the subplasmalemmal sites of the synaptic folds appeared under electron microscope as heavily contrasted zones (Fig. 2E).

Histochemical localization of AChE (Fig. 2G) or fluorescent bungarotoxin detection of AChRs (results not shown) indicated that the neuromuscular junctions (NMJs) were largely established in urodeles near the ends of myofibers which contained the MTJs, so that it is probable but difficult to assume that in these animals dystrophin is associated with the NMJs as well as with the MTJs.

Epimorphic regenerating muscle (blastema)

Epimorphic regeneration of newt tail skeletal muscle proceeds in a rostrocaudal gradient as does spinal cord or cartilage regeneration (Iten and Bryant, 1976; Khrestchatisky *et al.*, 1988; Thouveny *et al.*, 1991). Therefore, in longitudinal sections through newt tail regenerates, mature muscle fibers are seen in proximal myogenic zones whereas only condensed myoblasts or myotubes are present in distal regions (Thouveny *et al.*, 1991).

Sarcolemma dystrophin labeling was studied during this regeneration process by examining cross-sections through myogenic zones of 3 week-old regenerates. In distal parts of regenerates, young differentiating muscle cells showed a weak and discontinuous dystrophin staining pattern (Fig. 3A), whereas in proximal regions, older or mature muscle cells displayed a more regular or continuous labeling (Fig. 3B). In longitudinal sections through distal myogenic areas of regenerates, double immunofluorescence studies with anti-dystrophin and anti-laminin antibodies indicated that dystrophin and laminin appeared first concomitantly, and at this level, co-distributed at the anchoring terminal regions of myofibers (Fig. 3 D-E).

Tissue regenerating muscle (transition zone)

In the stump lesion region close to the amputation plane, a large part of the damaged skeletal muscle repairs itself by the tissue regeneration mode. In cross-sections through this transition zone adjacent to tail blastema or young regenerates — e.g. 2 week-old regenerates — most myofibers occurred unlabeled or weakly labeled with anti-dystrophin antibodies (Fig. 4A). Note that a punctate staining was frequently observed in the sarcoplasma of muscle cells which showed no peripheral dystrophin reactivity (Fig. 4C). Double immunostaining studies on cross-sections through injured skeletal muscle using anti-dystrophin and anti-laminin antibodies were also performed (Fig. 5A,B). In the transition zone close to young regenerates — e.g. 2 week-old regenerates — two populations of muscle fibers could be seen: (1) A population of myofibers which occurred organized in medial muscle masses and which showed dense myofibrillar material in phase-contrast images (Fig. 5C). These myofibers exhibited roughly similar immunostaining patterns with each of the two antibodies (Fig. 5A,B). (2) A population of myofibers which appeared distributed more laterally (Fig. 5C, asterisks) and which showed irregular and abnormal section profiles of damaged fibers. These muscle fibers displayed weak or no dystrophin labeling (Fig. 5A), whereas they showed an intense and ruffled laminin labeling (Fig. 5B) with respect to the regular profile of laminin reactivity in normal musculature. Electron microscopical observations (Fig. 6A,B,C) suggested that abnormal laminin staining pattern of severed skeletal muscle fibers (Fig. 6D), found in the transition zone, was probably due to the undulating feature of the old muscle basal lamina, which was largely detached from the sarcolemma (Fig. 6B,C).

Discussion

In this paper, the patterns of dystrophin expression were analyzed in mature skeletal muscle and during muscle epimorphic regeneration and tissue repair, which occurred in the regenerate and in the stump transition zone, respectively, after newt tail amputation. However, it is advisable to keep in mind that dystrophin reactivity in our immunoblotting and immunofluorescent studies could be due not only to authentic dystrophin but also to dystrophin-related proteins (DRP). Indeed, it is highly probable that the two anti-dystrophin Ab we used, which recognize the 400 kDa product of DMD gene, also cross-react with the 400 kDa product of the dystrophin-related gene (DMDL) linked to chromosome 6 (Love *et al.*, 1989; Khurana *et al.*, 1990). However, this fact was not a major obstacle for dystrophin immunostaining experiments on the skeletal muscle since specific antibodies raised against the DMDL protein localized DRP almost exclusively to the NMJs, DRP being undetectable in the nonjunctional sarcolemma (Man *et al.*, 1991; Ohlendieck *et al.*, 1991).

Dystrophin expression in adult skeletal muscle

Immunofluorescent studies on sections of adult skeletal muscle from newts have shown dystrophin uniform labeling of the sarcolemma as previously reported for normal human or mammal skeletal muscle (Arahata *et al.*, 1988; Bonilla *et al.*, 1988; Watkins *et al.*, 1988; Zubrzycka-Gaarn *et al.*, 1988; Carpenter *et al.*, 1990; Cullen *et al.*, 1990). Furthermore, in agreement with these studies and others more focused on MTJs (see e.g. Samitt and Bonilla, 1990), dystrophin immunostaining appeared more prominent at the MTJs, which were highly specialized sites for anchorage on connective tissue septa and bone processes and extensively folded regions with dense subsarcolemmal areas at which contractile actin fila-

Fig. 3. Expression of dystrophin and laminin in regenerating skeletal muscle of newts. *(A-B) Dystrophin staining pattern of differentiating muscle in cross-sections through 4 week-old tail regenerates of Nv.* **(A).** *Distal level of the regenerate. Immunoreactivity with H-5A3 MAb occurs weak and discontinous about the periphery of muscle (M) cells. X350.* **(B)** *More rostral myogenic region than in (A). Dystrophin labeling appears higher and more regular around most myofibers (M). Ep: Epidermis.* **(C)** *Phase-contrast image of (B). X350.* **(D-E)** *Double immunofluorescence staining of epimorphic regenerating myofibers in longitudinal section with anti-dystrophin and anti-laminin Ab.* **(D)** *Immunoreactivity with FITC-labeled anti-dystrophin H-5A3 MAb. The most intensely fluorescent parts of newly formed muscle fibers are seen at their terminal regions (arrows). Note that the rest of sarcolemma along the myofibers is not yet highly stained at this differentiation stage.* **(E)** *Immunoreactivity with TRITC-labeled anti-laminin PAb. Similar immunostaining pattern to that in (A) is observed.* **(F)** *Phase-contrast image of (A) and (B). X350.*

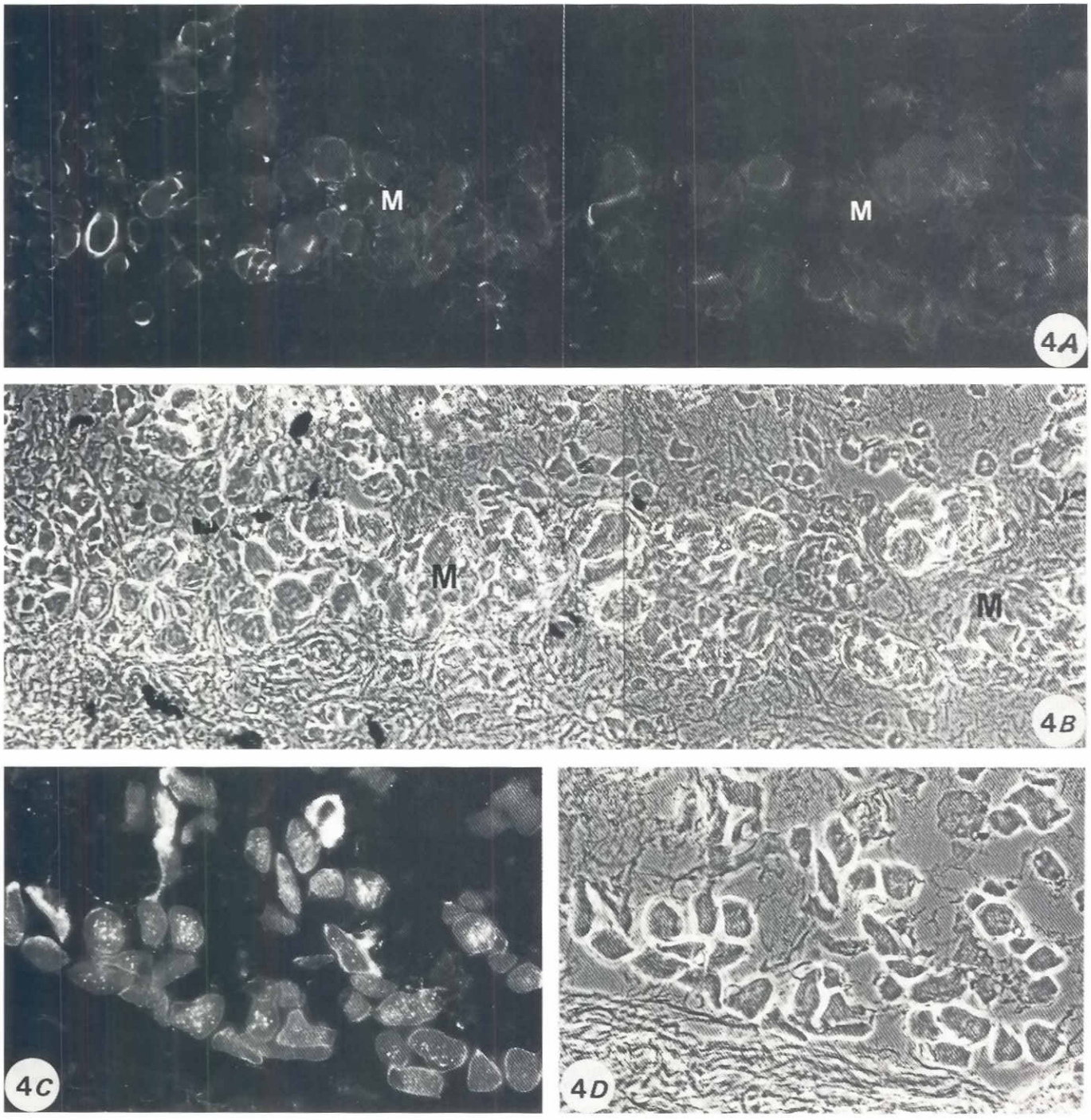


Fig. 4. Dystrophin labeling pattern with H-5A3 MAb in cross-sections through injured muscle of the Nv stump transition zone. (A) *Most muscle cells (M) appear weakly stained or unstained.* **(B)** *Phase-contrast image of (A). X350.* **(C)** *A punctate staining is observed here in the sarcoplasm of muscle cells which show only a little peripheral labeling.* **(D)** *Phase-contrast image of (C). X250.*

ments inserted. Samitt and Bonilla (1990) suggested that dystrophin could be one of the main components linking sarcomeric terminal actin filaments to the subplasmalemmal surface of the MTJs. This assumption was supported by electron microscopic observations on dystrophic MTJs of mdx mice (Tidball and Law, 1991), suggesting dystrophin is required for normal actin filament-membrane binding at the MTJs. It has been strongly suspected (Hammond, 1987; Davison and Critchley, 1988; Koenig *et al.*, 1988) and recently

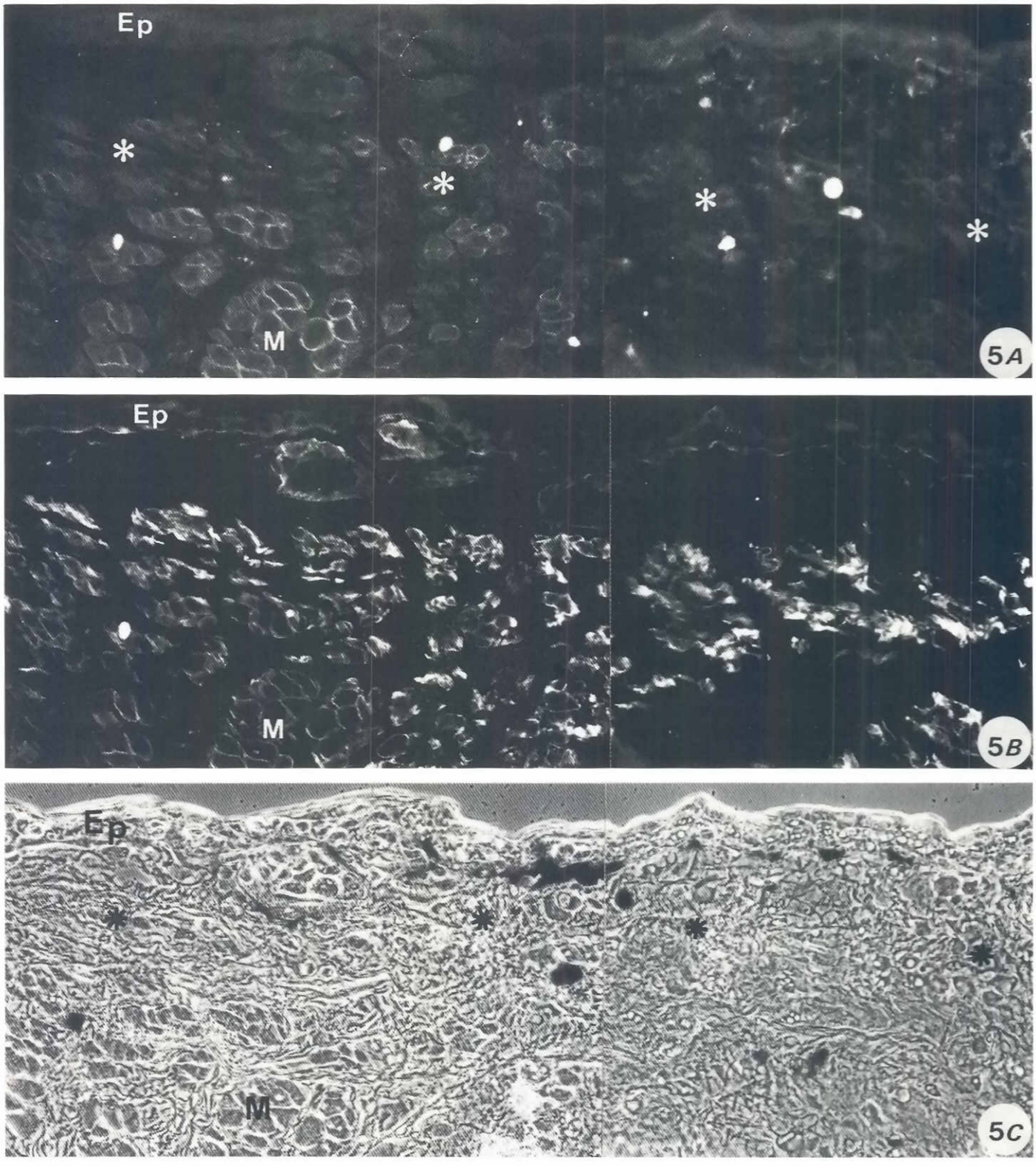


Fig. 5. Double staining pattern with anti-dystrophin and anti-laminin Ab in cross-sections through damaged muscle of the transition zone. (A) *Immunofluorescence with FITC-labeled anti-dystrophin H-5A3 MAb. Staining is observed around myofibers which appear organized in medial muscle masses (M) but weak or no staining is seen around injured fibers distributed more laterally (asterisks). Ep: epidermis.* **(B)** *Immunoreactivity with TRITC-labeled anti-laminin PAb. Dystrophin-positive fibers seen in (A) only show a slight laminin staining whereas dystrophin-deficient fibers in (A) exhibit an intense and ruffled laminin labeling.* **(C)** Phase-contrast image corresponding to (A) and (B). X250.

demonstrated (Hemmings *et al.*, 1992; Levine *et al.*, 1992; Way *et al.*, 1992) that dystrophin is a member of the superfamily of actin-crosslinking proteins that have, like alpha actinin and beta spectrin, homologous NH2-terminal actin-binding domains. The amino terminus of dystrophin may therefore be anchored to the myofibrillar cytoskeleton while the C-terminal end of the molecule is thought to link it to the plasma membrane via a large oligomeric complex of sarcolemmal glycoproteins (Campbell and Kahl, 1989; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991; Ibraghimov-Beskrovaya *et al.*, 1992).

On the other hand, histochemical detection of AChE and AChRs and previous electron microscopic investigations (Thouveny *et al.*, 1991) have shown that the NMJs are preferentially established in newts near the terminal areas of myofibers, which seems to be related to the metameric innervation pattern of the musculature seen in these animals (Thouveny *et al.*, 1991; Arsanto *et al.*, 1992). Therefore, it is highly probable that dystrophin/DRP reactivity observed at the ends of muscle fibers in urodeles not only concerns the MTJs but also the NMJs, as reported for those of human and mammal muscle (Fardeau *et al.*, 1990; Huard *et al.*, 1991; Man *et al.*, 1991; Ohlendieck *et al.*, 1991; Pons *et al.*, 1991). In addition, the more discrete NMJ synaptic dots present along the lateral surface of myofibers could be *a fortiori* masked by the strong dystrophin labeling associated with the sarcolemma.

Dystrophin expression during muscle epimorphic regeneration (blastema)

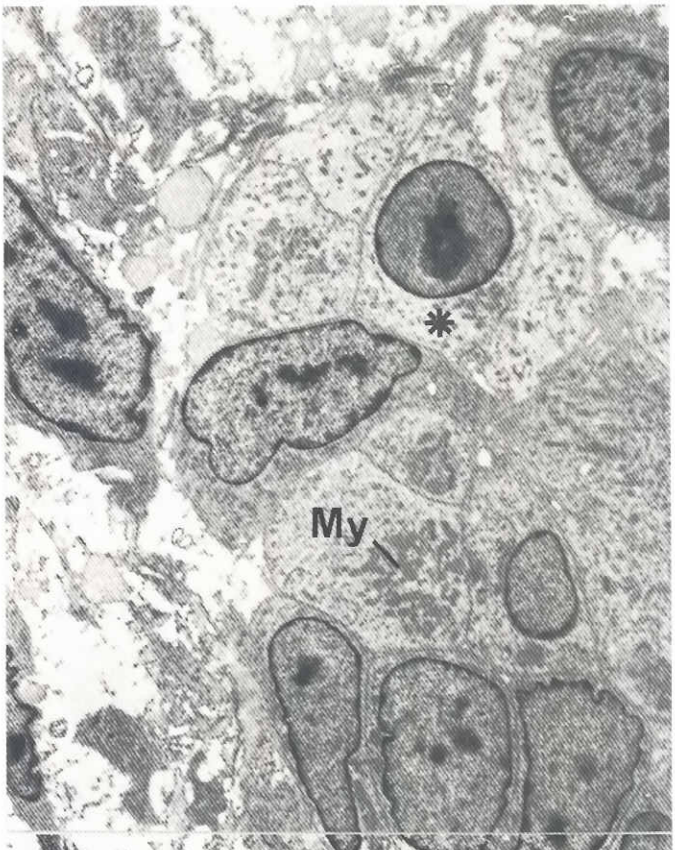
The epimorphic regeneration of skeletal muscle in newt tail regenerates — which proceeds in a rostrocaudal gradient — has been shown to be very similar to embryonic myogenesis (Iten and Bryant, 1976; Khrestchatisky *et al.*, 1988; Thouveny *et al.*, 1991) although the source of myoblasts was not yet known. The developmental expression pattern of dystrophin was examined with immunohistochemistry during this muscle regeneration process. We observed that dystrophin staining of sarcolemma was faint and discontinuous in fused myotubes and gradually extended to the whole plasma membrane in differentiated myotubes and myofibers. These findings are in agreement with those of Hagiwara *et al.* (1989) on embryonic and young rat skeletal muscle plasma membrane. They are also consistent with studies on cultured muscle cells of mammals, which have shown that dystrophin mRNA is expressed only in fused myotubes but not in myoblasts (Lev *et al.*, 1987; Nudel *et al.*, 1988), suggesting that dystrophin is regulated in a similar way to muscle-specific contractile proteins. However, using PCR to detect low-abundance transcripts in mouse myogenic cell lines, Chelly *et al.* (1990) have demonstrated that dystrophin mRNA is already expressed in myoblasts.

On the other hand, dystrophin staining experiments on longitudinal sections through distal myogenic areas of newt tail regenerates have shown that dystrophin first appears at the MTJ regions of the developing muscle fibers. This observation may be correlated with the fact that dystrophin labeling was first observed in human fetal muscle in the sarcoplasm zone near the MTJs (Wessels *et al.*, 1991). Furthermore, our double immunolabeling studies have shown that laminin of the basal lamina sheath first appears concomitantly with dystrophin at the terminal regions of myotubes and that the co-distribution of the two proteins then progresses from the end to the median part of fibers. The early and heavy accumulation of dystrophin at the MTJs is most probably related to the crucial role played by these structures in stabilizing the sarcolemma against the contraction stresses at these highly specialized sites for force transmission across the membrane.

Dystrophin expression in muscle tissue repair (transition zone)

In Amphibian urodeles, the damaged skeletal muscle fibers in the stump lesion transition region close to the amputation plane largely repairs itself by a tissue regeneration mode (Carlson, 1979; Thouveny *et al.*, 1991). In mammalian muscle regenerating in this way, the myogenic reserve cells which replace the necrosed injured fibers are satellite cells present beneath the muscle basal lamina (for review, see e.g. Carlson, 1979). Authentic satellite cells have never been observed in adult newt muscle (Hay, 1962; Lentz, 1969; Iten and Bryant, 1976). Cameron *et al.* (1986) proposed as a source of regenerated adult newt muscle *in vitro* post-satellite cells resembling fibroblasts, each of them being enveloped in its own external lamina. These data suggest that the modalities of muscle tissue repair cannot therefore be exactly the same in newts and in mammals. On the other hand, on the basis of electron microscopic investigations, many workers (Hay, 1962; Lentz, 1969; Iten and Bryant, 1976) have proposed the hypothesis that myoblasts in regenerating adult urodele appendages arise through dedifferentiation of mature myofibers. We think that muscle cells exhibiting weak or no dystrophin labeling in the damaged skeletal muscle of the stump transition zone might be fully or partially dedifferentiated cells rather than differentiating post-satellite cells. Indeed, the ruffled laminin staining pattern seen around muscle cells in this region probably visualized the remnants of the old basal lamina sheath of severed fibers and not a newly basal lamina around developing myofibers. Electron microscopic studies supported this view by showing the undulating feature of the muscle basal lamina which occurred more or less largely detached from the sarcolemma. Moreover, it is unlikely that all injured muscle fibers of the stump transition zone undergo complete and irreversible degeneration since fully developed morphological signs of necrosis (e.g. disappearance of the plasma membrane) with macrophage invasion were rarely observed. In agreement with Carpenter and Karpati (1979), who reported similar observations for DMD dystrophinless fibers, our findings suggest that dystrophin may be involved in anchorage between the plasma membrane and the basal lamina. Recent data of Ibraghimov-Beskrovaya *et al.* (1992)

Fig. 6. Electron micrographs of cross-sections through areas of severed muscle in the transition zone close to the amputation level of Nv tail. **(A)** *Most muscle cells which appear mononucleate show a loosely organized or disrupted myofibrillar system (My). One of these cells (asterisk) obviously displays a rounded nucleus with prominent nucleolus. Note also the poor degree of organization of muscle connective tissue. C: collagen fibrils. X2700.* **(B-C)** *Enlarged views of injured myofibers (M) in which the largely ruffled feature of their basal lamina (BL) sheath can be seen. Only threads (small arrows) of it still occur in normal close apposition to the cell surface. Note the presence of collagen fibrils (arrowheads) on fibroblasts (F) located in the vicinity of the myofibers. My: myofibrils; Z: Z band. X6750.* **(D)** *Laminin staining pattern of damaged myofiber BL, the undulating feature of which is seen in (B) and (C). X250.*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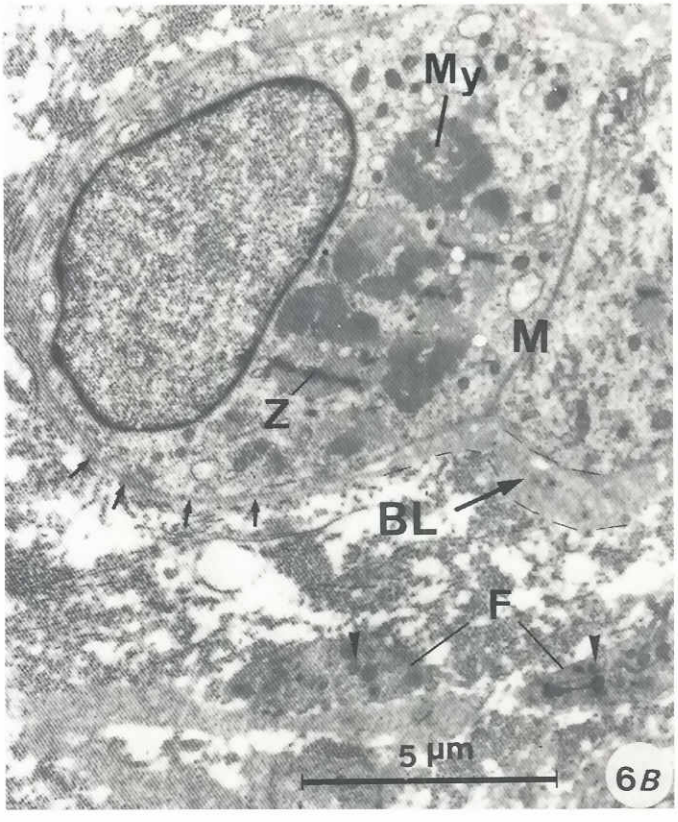
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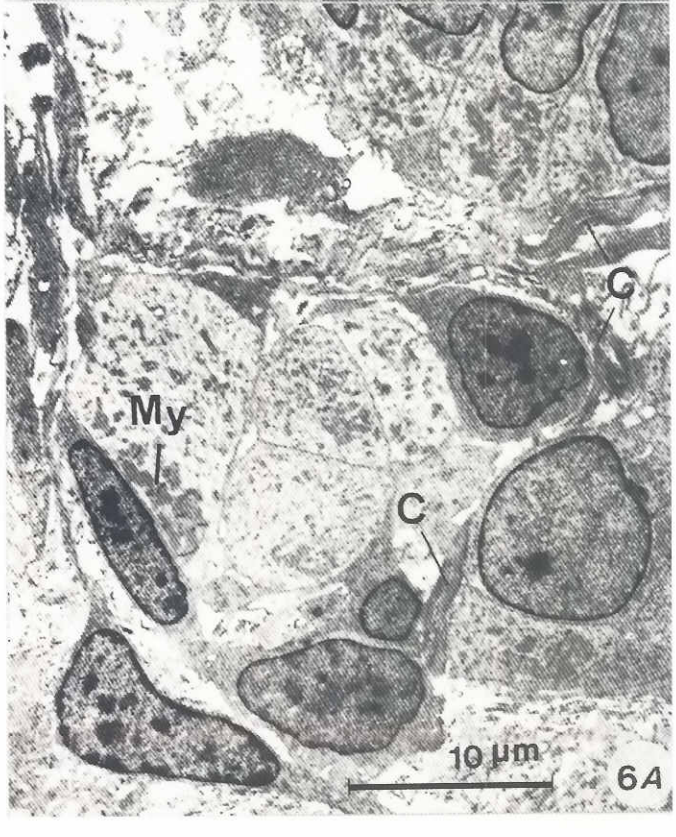
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have shown that the 156 kDa dystrophin-associated glycoprotein specially binds laminin, and thereby could provide a mediated dystrophin-laminin linkage between the subsarcolemmal cytoskeleton and the basal lamina. Interestingly, the close temporal and spatial relationship between dystrophin and laminin at the ends of epimorphically regenerating myotubes is also consistent with the idea that the two proteins may play a determinant role in sarcolemma-extracellular matrix interaction.

In conclusion, this study reported for the first time patterns of dystrophin expression in adult and regenerating tail skeletal muscle of Amphibian urodeles. Further experiments are now needed to ascertain whether muscle tissue repair near the amputation zone really implicate a dedifferentiation process although a participation of post-satellite cells as source of myoblasts could not be ruled out.

Materials and Methods

Animal surgical procedures

The urodelean amphibia used in this study were adult newts: *Pleurodeles waltl* (Pw) obtained from the C.N.R.S. Amphibian farm, Centre de Biologie du Développement, Université Paul Sabatier, Toulouse, France; *Notophthalmus viridescens* (Nv), from Lee's Newt Farm, Oak Ridge, Tennessee. Animals were reared in groups of 10-12 and maintained in circulating tap water thermostatted at 18-20°C; the water was completely renewed twice a week. Pw were fed twice a week with beef heart or liver, and Nv with tubifex. Before surgery, animals were anesthetized with 1:1000 MS 222 (tricaine methane sulfonate, Sigma). Amputations were performed in the third distal part of the tail, at the level of intervertebral junctions. Operated animals were observed daily. After appropriate periods of regeneration, the blastema were harvested by reamputation.

Antibodies

Various recombinant proteins, constructed from fragment of chicken skeletal muscle dystrophin cDNA (Lemaire *et al.*, 1988) were used to produce specific dystrophin antibodies.

Polyclonal antibody (PAb) directed against dystrophin sequence «1173-1728» was later named C as previously described (Augier *et al.*, 1992).

Anti-dystrophin monoclonal antibody (MAb) H-5A3 corresponding to residues 3357-3660 was obtained as described in Harricane *et al.* (1991). The specificity of this dystrophin MAb towards Xp21 and 6q24 dystrophin forms was demonstrated by epitope mapping (manuscript in preparation).

Anti-mouse laminin PAb used with anti-dystrophin H-5A3 MAb in double-labeling experiments was a gift of Dr. J-C. Lissitzky.

SDS-PAGE and Immunoblot analysis

Cross-reactivity of antibodies with urodele amphibian antigens was controlled by Western blots. Immediately after dissection, samples (skeletal muscle, smooth muscle, brain, spinal cord and tail regenerates) were homogenized in 7 volumes of 20 mM, Tris-HCl, 10 mM EDTA, 0.2% Triton x100, 1 mM Phenyl Methyl Sulfonyl Fluoride (PMSF), 1 µg/ml antipain and pepstatin 15 µg/ml, benzamidine pH 8 (antipain, pepstatin and benzamidine were first solubilized in dimethylsulfoxide (DMSO). The homogenization was carried out on ice in a Dounce homogenizer with a glass pestle for young regenerates, and muscle tissues and older regenerates were crushed in an Ultra-Turrax T25 tissue grinder for 15-20s before being homogenized. The samples were then sonicated and centrifuged at 10,000g for 10 min at 4°C. Proteins present in the supernatant were resolved on 7% or 5-13% sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). Proteins were then transferred onto nitrocellulose membrane according to Burnette (1981).

The nitrocellulose sheets were sequentially reacted with primary Ab (used without dilution for the MAb H-5A3 and at a 1:50 dilution for the PAb), and then with alkaline phosphatase-coupled second antibody (at 1:500). BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate) and NBT (Nitroblue Tetrazolium) were used for development of phosphatase activity.

Fluorescence microscopy

Samples were directly embedded unfixed in OCT and frozen in liquid nitrogen. Longitudinal and cross sections of 15 µm were cut in a cryostat at -22°C. They were collected on gelatined slides and stored at -20°C. They were washed 1 h in PBS +1% bovine serum albumine (BSA), then incubated 1 h with primary antibodies used without dilution for anti-dystrophin MAb H-5A3 or at a 1:40 dilution for anti-dystrophin PAb. Fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG or anti-rabbit IgG were used at a 1:100 or 1:200 as secondary antibodies.

Furthermore, in order to compare the distribution of dystrophin with that of laminin or AChRs, double staining experiments were performed using anti-dystrophin PAb or MAb H-5A3 labeled with tetramethyl rhodamine isothiocyanate (TRITC) and FITC-conjugated anti-mouse-laminin PAb or alpha-bungarotoxin (1 to 10 µg/ml; Sigma).

Washed slides were mounted in moviol and observed with epifluorescence Zeiss microscope and photographed on Tri-X pan (Kodak). Controls were made by omitting the first antibody or by replacing it with preimmune serum.

Histochemistry

For the detection of acetylcholinesterase (AChE) sites, samples were directly embedded unfixed in OCT (Miles Scientific, Naperville, Il) and frozen in 2-methyl- butane cooled with dry ice. Serial cryostat sections cut at 15 µm were fixed and stained with the Goshgarian (1977) silver-AChE method for nerve fiber and end-plate detection and counterstained with eosin.

Electron microscopy

Tissue samples were fixed for 2.5 h in 2.5% glutaraldehyde, 4% formaldehyde in 0.1M cacodylate buffer (pH 7.4), postfixed for 1 h in osmium tetroxide in the same buffer, dehydrated in acetone and embedded in araldite. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with Hitachi H600 electron microscope.

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